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Comparative Effects of *Azospirillum brasilense* Sp245 and *Pseudomonas aeruginosa* PAO1 Lipopolysaccharides on Wheat Seedling Growth and Peroxidase Activity

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Abstract
The effects of lipopolysaccharides (LPS) from *Azospirillum brasilense* Sp245, a plant growth-promoting rhizobacteria, and *Pseudomonas aeruginosa* PAO1, a pathogenic bacterium, on plant growth and peroxidase (POD) activity were assessed on wheat seedlings. *A. brasilense* LPS (100 µg/mL) increased total length, and total fresh weight in wheat seedlings 4 days after treatment. *P. aeruginosa* LPS did not show effect on plant growth. *A. brasilense* LPS increased root hairs length similar to whole cells, while *P. aeruginosa* LPS increased root hairs density and slightly root hairs length. Both LPS increased POD activity and hydrogen peroxide (H_2O_2) content in root; however, the LPS from the pathogenic bacterium generated higher increments. The peroxidase inhibitor salicylhydroxamic acid (SHAM) inhibited plant growth, which was not recovered by the addition of LPS neither *A. brasilense* nor *P. aeruginosa*. POD activity stimulated by LPS was calcium-dependent as confirmed by the addition of the calcium channel blocker $LaCl_3$. The results suggest that plant cells sense differentially LPS from beneficial or pathogenic bacteria and that calcium is needed to respond to the presence of both LPS.

Keywords *Azospirillum brasilense* · *Pseudomonas aeruginosa* · Lipopolysaccharides · Wheat · Peroxidase · Plant growth

Introduction

Lipopolysaccharides (LPS) are major components of the outer membrane (OM) of Gram-negative bacteria that contribute to membrane integrity and stability. LPS also contribute to cell defense against external stress factors by providing a permeability barrier against many different classes of molecules including antibiotics and metals (Silipo and Molinaro 2017; Molinaro et al. 2009).

LPS are involved in adhesion and colonization in host-bacterium interactions. In addition, LPS are potent elicitors of innate immune responses during pathogenesis of Gram-negative infections in both plant and animal hosts (Takeuchi and Akira 2010; Ranf 2016; Ranf et al. 2016). All LPS share three common structural components: a lipophilic moiety termed lipid A, a core oligosaccharide, and an O-specific polysaccharide (also known as O-chain or O-antigen), a hydrophilic glycan. The core oligosaccharide contains a carbohydrate that is specific to LPS: 3-deoxy-D-*manno*-oct-2-ulosonic acid (Kdo), as well as several heptose moieties. The O-specific polysaccharide is joined to the Kdo domain and is oriented outwards, whereas lipid A is embedded in the OM to anchor LPS to the membrane (Whitfield and Trent 2014).

LPS from plant pathogens trigger the metabolomics reconfiguration of primary and secondary metabolites synthetized from various metabolic pathways (Mareya et al. 2020). Furthermore, the intact LPS structure is required for its complete biological activity, as the lipid A and the polysaccharide (O-antigen-core) chain reprogramming cellular activities at low levels than intact LPS (Madala et al. 2012). Although, the lipid A alone is able to reprogram the cellular metabolism related with immunity and defense (Madala et al. 2011).

Gram-negative bacteria that contain LPS include the plant growth-promoting rhizobacteria (PGPR) *Azospirillum brasilense* and the opportunistic pathogen *Pseudomonas aeruginosa*. *Azospirillum* is the most studied PGPR and is a widely used model of bacterial interactions with many plant species

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(Cassán et al. 2020; Pereg et al. 2016; Steenhoudt and Vanderleyden 2000). *A. brasilense* LPS are highly active in interactions with plant roots (Fedonenko et al. 2001; Matora et al. 1995) and in induction of plant responses (Evseeva et al. 2011). Furthermore, *A. brasilense* Sp245 LPS can promote wheat plant growth in vitro (Vallejo-Ochoa et al. 2018) and under greenhouse conditions (Chavez-Herrera et al. 2018).

Pseudomonas is a bacterial genus that has a wide distribution and these bacteria inhabit various ecological niches. As a phytopathogenic bacteria, *Pseudomonas* colonize surfaces of plant leaves to form dense bacterial populations that do not cause disease due to an inability to penetrate the leaf epidermis directly, but rather enter into plant tissue through natural surface openings such as stomata or wounds (Gimenez-Ibanez and Rathjen 2010; Starkey and Rahme 2009).

Pseudomonas aeruginosa is an opportunistic pathogen of plants and is ubiquitously distributed in soil and aquatic habitats (Sitaraman 2015). The *P. aeruginosa* strain PAO1 is the most commonly used strain for research on this opportunistic pathogen, and has become the reference strain to study the genetics, physiology and metabolism of this gamma proteobacterium (Chahtane et al. 2018; Klockgether et al. 2010). *P. aeruginosa* can also infect plant roots and thus is also used to study the plant defense responses against bacterial pathogens (Walker et al. 2004).

Although LPS have been isolated from some *Pseudomonas* strains, for example from *P. aeruginosa* PAC1 (Chester and Meadow 1975), their effects on plant growth and some biochemical responses such as peroxidase activity are unknown.

Peroxidases (PODs, E.C. 1.11.1.7) are hemoproteins expressed by a range of organisms. These enzymes catalyze the oxidation of numerous substrates using hydrogen peroxide and are involved in a variety of biological processes (Kalsoom et al. 2015). Class III PODs are specific to plants and are key proteins that control plant differentiation and development (Renard et al. 2020; de Carvalho Oliveira et al. 2019; Yan et al. 2019; Francoz et al. 2015). They belong to large, multigenic families with 73 members reported for *A. thaliana*, 181 for *Eucalyptus grandis*, 138 for *Oryza sativa* and 143 for *Brachypodium distachyon* (Fawal et al. 2013).

Different PODs have antagonistic activities that contribute to the loosening and stiffening of the cell wall that occurs during plant growth and cell expansion. To stiffen the cell wall, PODs oxidize aromatic compounds in the cell wall such monolignols and cinnamic acids in the presence of H_2O_2 to promote covalent bond formation. To loosen the cell wall, PODs generate radical oxygen species (ROS) such as hydroxyl radical (OH^-) that break covalent bonds in cell wall polymers (Schopfer 2001). PODs can also control cell elongation through their auxin oxidase activity (Cosio et al. 2009). Plant PODs contain two calcium ions (Ca^{2+}) that are critical for maintaining structural stability around the heme moiety and also for preserving thermal stability (Maranon and Huystee 1994; Shiro et al. 1986).

POD activity is induced by *A. brasilense* in inoculated *Handroanthus impetiginosus* shoots (Larraburu et al. 2016) and by *Pseudomonas* in wheat plants (Minaeva et al. 2018). However, less is known about how LPS of these bacteria affect POD activity. In this work, we compared the effect of LPS from *A. brasilense* Sp245 and *P. aeruginosa* PAO1 on early growth and root peroxidase activity in wheat plants.

Materials and Methods

Materials and Growth Conditions

The strains *A. brasilense* Sp245 (Baldani et al. 1986) and *P. aeruginosa* PAO1 (Klockgether et al. 2010) were used in this study. Both strains were routinely grown on solid LB medium (10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl, 0.186 g/L $MgSO_4$, 0.277 g/L $CaCl_2$, 15 g/L agar). To prepare inocula, cultures were grown on liquid LB medium for 16 h (exponential phase) at 27 °C with shaking at 100 rpm. The cultures were then washed twice in 0.9% NaCl by centrifugation (4300×*g*, 10 min, 4 °C), resuspended in sterile water, and adjusted to a final concentration of 10^6 colony-forming units (CFU)/mL.

Triticum aestivum seeds, cv Nana F2007, were kindly provided by Dr. Mario González-Chavira (Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecuarias—Celaya, Guanajuato, México). For germination, seed were washed with 1% sodium dodecyl sulfate (SDS) for 3 min. The seeds were then surface sterilized with 1% sodium hypochlorite for 5 min and rinsed four times with sterile distilled water. The seed were place in a petri dish containing a wet sterile filter paper and incubated in the dark at 28 ℃ for 3 days.

Inoculation and Treatments

After germination, seedlings were transferred to sterilized test tubes (15 cm long and 2 cm wide) and only the roots were immersed in 10 mL liquid Murashige and Skoog (MS) medium (pH 5.7). Inoculation with the bacteria was performed by adding 10^6 CFU/mL in MS medium. For LPS treatment, LPS was added to the liquid medium at two different concentrations (10 or 100 µg/mL). Four days after the LPS treatments, plant growth was assayed in terms total fresh weight as well as the length of leaves, roots, and total plant length. Images of the root were recorded with a stereomicroscope (Leica MZ6).

Salicylhydroxamic acid (SHAM; 100 µM), lanthanum chloride ($LaCl_3$; 0.5 mM), and calcium chloride ($CaCl_2$; 100 µM) were obtained from Sigma-Aldrich (St. Louis, MO,

USA). Chemicals were added to MS medium at the indicated concentrations 30 min before the addition of LPS.

Extraction of Bacterial LPS

LPS extraction was performed as previously described (Renukadevi et al. 2012). Cell wall fractions were isolated from 50 mL of a culture grown for 16 h in liquid LB medium (27 °C). Cells were first pelleted by centrifugation at 4000×*g*, for 10 min and resuspended in 5 mL sterile distilled water with 5 mL phenol equilibrated with 10 mM Tris–HCl, pH 8 and incubated at 65 °C for 20 min with shaking. The samples were then incubated at 4 °C for 24 h. The crude LPS were then purified by dialysis for 3 days at 5 °C against deionized water. The LPS were concentrated by alcohol precipitation wherein sodium acetate was added to a final concentration of 0.15 M followed by dropwise addition of ice cold 96% ethanol to yield a final ethanol proportion of 1:4. The mixture was incubated for 24 h at − 20 °C. The pellet was then collected by centrifugation at 4000×*g* for 20 min and suspended in distilled water. LPS were stored at 4 °C until use.

SDS-PAGE and LPS Silver Staining

Preparations were developed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli 1970). LPS preparations were separated on an SDS-PAGE gel with 4% and 12.5% acrylamide in the stacking and separating gels, respectively. Electrophoresis was performed at 12 mA through the stacking gel and 25 mA in the separating gel.

The LPS silver staining procedure was modified from Fomsgaard et al. (1990). Gels were fixed overnight in 100 mL 40% (vol/vol) ethanol in 5% (vol/vol) acetic acid. The gels were then subjected to a 5 min oxidation in 100 mL 0.7% periodic acid, 40% (vol/vol) ethanol, 5% (vol/vol) acetic acid. After oxidation, the gels were washed four times for 1 h each with 100 mL distilled water before incubation for 10 min in freshly prepared silver staining solution consisting of 18.66 mL 0.1 N sodium hydroxide, 1.33 mL 29.4% ammonium hydroxide and 3.33 mL 20% (wt/vol) silver nitrate, with distilled water added to yield a final volume of 100 mL. After staining, the gels were washed three times for 15 min each with 100 mL distilled water. The gels were then developed for 10 to 20 min by incubation in 100 mL freshly prepared developer solution consisting of 5 mg citric acid and 0.05 mL 37% formaldehyde, in 100 mL distilled water, at 37 °C. After visualizing the LPS bands, the gel was transferred to a stop bath (100 mL 0.35% [vol/vol] acetic acid in distilled water) and incubated for 1 h. Finally, the gel was washed with 100 mL distilled water and stored in sealed plastic bags with a small amount of water to prevent dehydration. Gel images were recorded with a digital camera (C-3030 Zoom, Camedia, Olympus, Tokyo, Japan).

Total POD Assay

Soluble POD assays were performed according to Svalheim and Robertsen (1990). Roots (~ 100 mg) were ground with a mortar and pestle in liquid nitrogen and the resulting powder was resuspended in 10 mM sodium phosphate, pH 6.0 and homogenized using a vortex. The suspension was assayed for POD activity by the formation of tetraguaiacol measured as an increase in absorbance at 470 nm in a spectrophotometer (Beckman Instruments, Fullerton, CA). Each reaction mixture (1 mL) consisted of 10 µL root extract and 990 µL guaiacol solution containing 0.25% guaiacol (v/v) in 10 mM sodium phosphate buffer pH 6.0 and 0.125% H_2O_2 (v/v). The reaction was monitored for 1 min. The protein content of the extracts was determined (Bradford 1976) using the Bio-Rad Protein Assay dye reagent (Bio-Rad, Hercules, CA). Bovine serum albumin was used as the standard.

For in situ detection of POD, wheat roots were transferred to 20 mL of medium containing 0.1 M Tris–acetate (pH 5), 0.1 mM 2,6-dichlorophenolindophenol (2,6-DCPI), 0.9 mM H_2O_2, at pH 5. After a 30 min incubation at room temperature, a colorimetric reaction occurred in the tissue (Córdoba-Pedregosa et al. 2003). The root was immediately photographed using a stereomicroscope.

H_2O_2 Analysis

The H_2O_2 content was assayed according to a modified procedure described by García-Pineda et al. (2010). Root tissue (100 mg) was powdered in liquid nitrogen and homogenized in 1.5 mL of deionized water. The samples were centrifuged at 4000×*g*, for 5 min. H_2O_2 was measured by incubating 500 µL of the supernatant with an equal volume of a reagent mixture containing 0.05% guaiacol (440 µL/L) and horseradish peroxidase (350 µL/L; 2,500 U/mL) dissolved in 25 mM sodium phosphate buffer (pH 7.0). The mixture was incubated for 15 min at room temperature in the dark and then the absorbance was measured immediately at 450 nm. Commercial H_2O_2 was used to generate a standard curve.

H_2O_2 localization in the root was assayed according to the procedure described by Thordal-Christensen et al. (1997). Roots were placed in 1 mg/mL 3,3′-diaminobenzidine (DAB), pH 3.8 and incubated in the dark for 2 h before transfer to 96% ethanol and analysis with a stereomicroscope.

Data Analysis

Experiments were repeated at least three times and data are expressed as mean ± standard error (SE). Statistical analyses were done with one-way analysis of variance (ANOVA)

followed by Duncan's multiple range tests for independent samples. In all cases, the confidence coefficient was set at $p < 0.05$.

Results

Effect of LPS on Root Morphology and Plant Growth

LPS from both *A. brasilense* and *P. aeruginosa* were extracted and verified by gel electrophoresis (Fig. 1a). The band pattern obtained was different to LPS from the two bacteria, suggesting structural differences of the molecules. Seedlings 3 days after germination were incubated with LPS at 10 or 100 µg/mL for 4 days and the effect on plant growth and root morphology was then compared. LPS from *A. brasilense* at 100 µg/mL increased the total length and total fresh weight of the seedlings. In contrast, LPS from *P. aeruginosa* at either concentration had no effect on these growth parameters (Fig. 1b). The effect of LPS treatment (100 µg/mL) and the bacteria (10^6 CFU/mL) on root morphology, specifically on the formation of hair roots, was visually compared. Both types of bacteria induced an increase in hair root length compared to uninoculated seedlings. Interestingly, *A. brasilense* LPS increased hair root length by a similar amount as that induced by incubation with whole cells, whereas root hairs of plants treated with *P. aeruginosa* LPS were shorter and more dense compared to that seen for *A. brasilense* LPS (Fig. 1c).

The effect of both types of bacteria and both types of LPS on leaf length and root length was next compared. For *A. brasilense*, exposure to whole cells resulted in a marked decrease in root length, whereas root length increased following treatment with *A. brasilense* LPS. Meanwhile, no change in root length was observed with either whole *P. aeruginosa* cells or with only *P. aeruginosa* LPS (Fig. 2).

POD Activity and H_2O_2 Production in Roots in Response to Different LPS

POD activity was analyzed in root seedlings after treatment with 100 µg/mL LPS from either *A. brasilense* or *P. aeruginosa*. The enzyme activity of PODs analyzed 4 days after treatment increased following treatment with both types of LPS relative to untreated control seedlings, although a larger increase was seen for LPS from *P. aeruginosa* compared to that from *A. brasilense*. An in situ analysis using 2,6-DCPI showed that the POD content increased in root tips

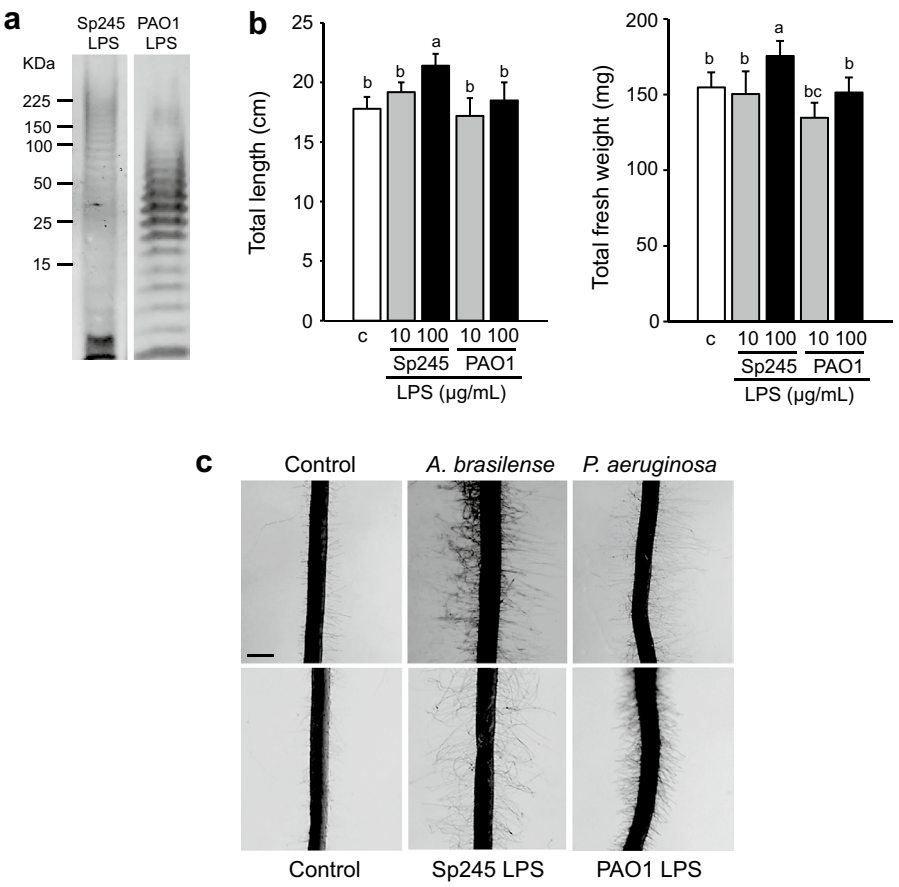

Fig. 1 Effect of LPS on plant growth and root morphology in wheat seedlings. **a** Analysis of LPS on SDS-PAGE. **b** Analysis of different concentrations of LPS on plant growth. **c** Hair root analysis after LPS (100 µg/mL) treatment. Barr = 1 mm. Data are mean ± SE of three independent experiments ($n = 10$). Letters above bars indicate significant differences according to a Duncan test ($p < 0.05$)

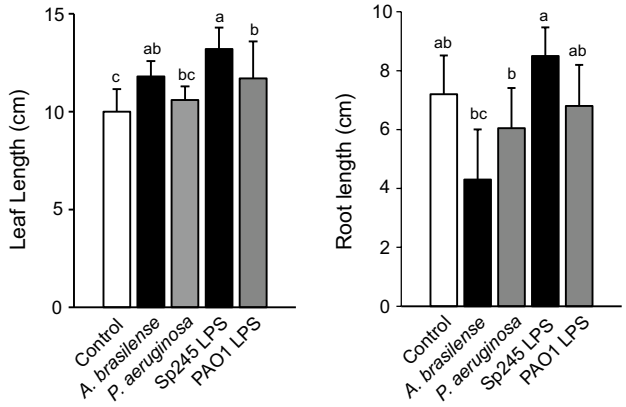

Fig. 2 Comparative effects of bacteria (10^6 CFU/mL) and LPS (100 µg/mL) on wheat seedlings growth. Data are mean ± SE of three independent experiments ($n = 10$). Letters above bars indicate significant differences according to a Duncan test ($p < 0.05$)

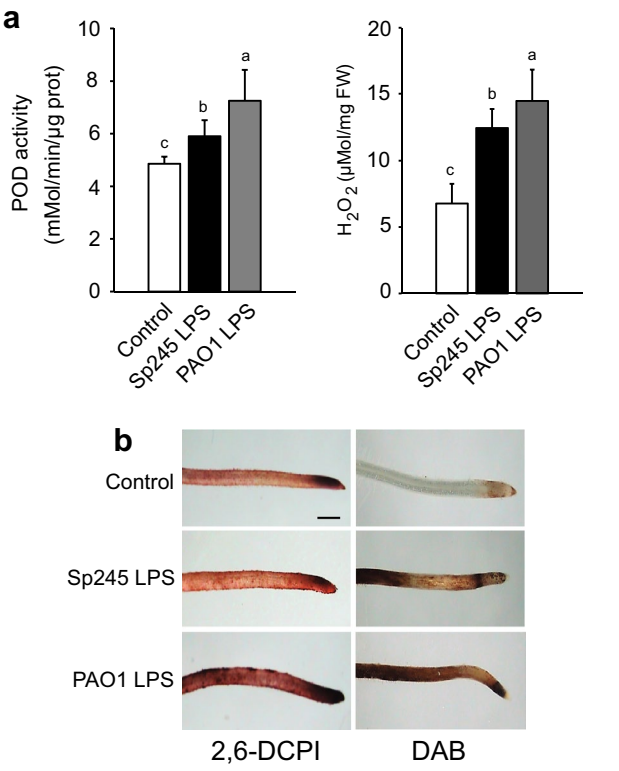

Fig. 3 Effect of LPS (100 µg/mL) on POD activity and H_2O_2 production in wheat root. **a** Wheat seedlings were treated with different LPS and after 4 days POD activity and H_2O_2 content were analyzed in roots. **b** In situ detection of POD and H_2O_2 in roots. 2,6-DCPI = 2,6-dichlorophenolindophenol; DAB = 3,3′-diaminobenzidine. Barr = 1 mm. Data are mean ± SE of three independent experiments ($n = 10$). Letters above bars indicate significant differences according to a Duncan test ($p < 0.05$)

but the increase was more notable for seedlings treated with *P. aeruginosa* LPS compared to those exposed to *A. brasilense* LPS, suggesting that the biochemical response varied according to LPS type. In addition, the different LPS had varying potential to increase the POD content, which correlated with the levels of total POD activity (Fig. 3a).

H_2O_2 production in roots as a biochemical response to LPS treatment was also analyzed. Treatment with both types of LPS notably increased the accumulation of H_2O_2 relative to untreated seedlings, but the accumulation was higher for plants treated with LPS from *P. aeruginosa* compared to that from *A. brasilense*. An in situ analysis of the location and content of H_2O_2 in root using DAB showed a similar pattern to that observed for POD enzymes, wherein *P. aeruginosa* LPS more effectively stimulated H_2O_2 accumulation (Fig. 3b).

The role of POD in responses stimulated by LPS was next analyzed using the POD inhibitor SHAM. Seedlings treated with 100 µM SHAM followed by 100 µg/mL LPS showed a decrease in plant growth. This effect was most clearly manifested in root length and weight (Fig. 4a). The same effect of LPS was observed for POD activity in roots, suggesting that this enzyme could mediate the biological activity of LPS (Fig. 4b).

Effect of Calcium on Plant Responses Stimulated by LPS

The role of calcium on responses stimulated by LPS treatment was analyzed with $LaCl_3$, a calcium channel blocker, as well as by the addition of $CaCl_2$. Increases in total length and total fresh weight of the seedlings and POD activity in the root were inhibited with the addition of the channel blocker and were not recovered following LPS treatment. In contrast, these parameters were increased in a similar manner to that seen for stimulation with LPS when calcium was added, but the increases were not additive with those seen in presence of LPS (Table 1). These results suggested that LPS requires calcium mobilization to stimulate the observed cellular responses.

Discussion

There is limited information concerning whether plants exhibit different responses to LPS from beneficial or pathogenic bacteria. In the present study, we compared several morphological and biochemical responses of wheat seedlings to LPS isolated from one type of beneficial rhizobacteria, *A. brasilense*, and one opportunistic plant pathogen, *P. aeruginosa*. LPS from *A. brasilense* dose-dependently increased plant growth and root hair length in a manner similar to that seen for incubation with whole cells, indicating that these molecules play a role in stimulating

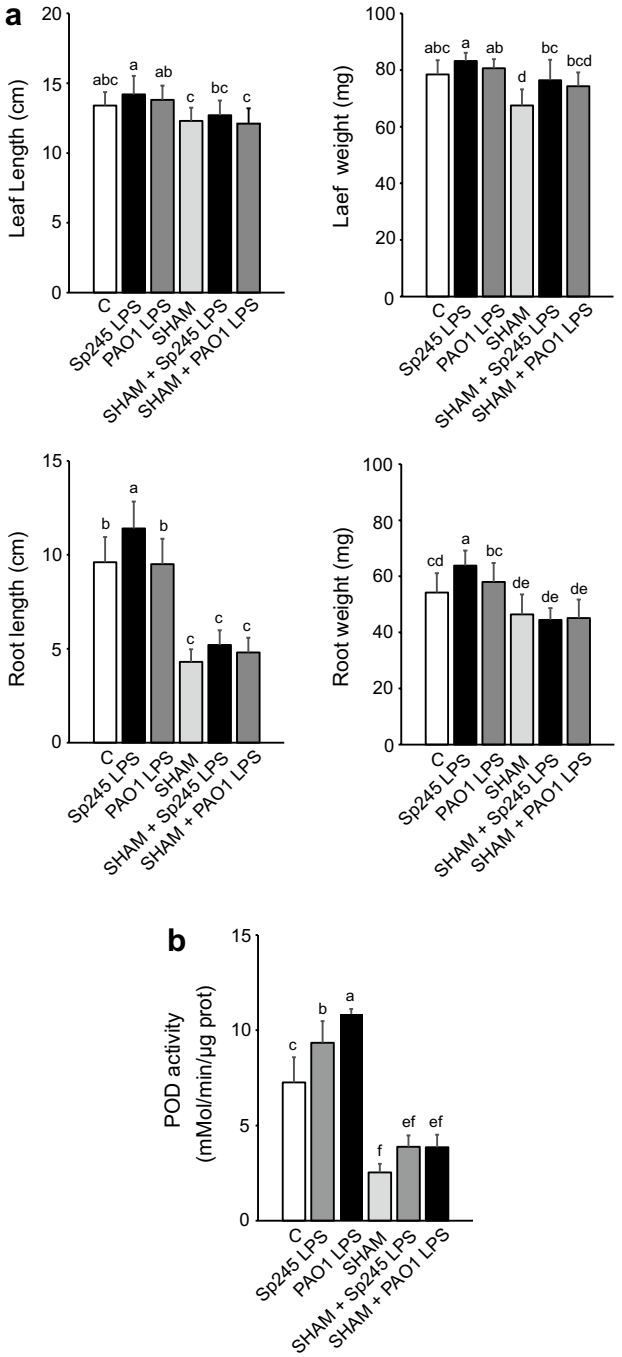

Fig. 4 Effect of SHAM on plant growth and POD activity. Wheat seedlings were treated with SHAM (100 µM), incubated by half hour and then LPS (100 µg/mL) were added. Plant growth (**a**) or POD activity (**b**) were analyzed 4 days after treatment. Data are mean ± SE of three independent experiments ($n = 10$). Letters above bars indicate significant differences according to a Duncan test ($p < 0.05$)

changes in root morphology that could drive plant growth. Meanwhile, neither concentration of LPS from *P. aeruginosa* exerted a stimulatory effect on plant growth or root hair length.

Results of previous reports indicated that phytohormones, such as auxins, gibberellins and cytokinins produced by PGPR are required for root morphogenesis (Ambreetha et al. 2018; Zaidi et al. 2015). However, in this study we showed that LPS alone derived from the PGPR *A. brasilense* could induce changes in plant growth and root morphology. The results suggest that *A. brasilense* can alter root morphology via structural molecules such as LPS, in addition to phytohormones production. The results also showed that plant roots react differently to LPS derived from a pathogenic microorganism in that LPS from *P. aeruginosa* did not affect plant growth.

In contrast to a decrease in root length observed upon incubation of seedlings with *A. brasilense* cells, treatment with *A. brasilense* LPS was associated with increased root length. Although the reason for this contrasting result is unclear, it does suggest that phytohormones produced by rhizobacteria mediate decreases in root length, as was previously reported (Cassan et al. 2014). Phytohormones produced by rhizobacteria could mask the potential effects of LPS during the interaction between plant and bacteria, and it is possible that LPS must directly contact root tissue to exert a stimulatory effect.

POD activity is stimulated by both bacterial plant pathogens (Farahani and Taghavi 2016; Jang et al. 2004) and beneficial rhizobacteria (Lavania et al. 2006), but less is known about how LPS from these bacteria affect POD activity. Here we compared the effect of LPS on POD activity and found that while both types of LPS increased POD activity, LPS from the pathogenic bacteria were associated with larger increases in POD activity, suggesting that plants may be able to distinguish between LPS from PGPR and bacterial pathogens.

PGPR LPS provide beneficial effects for plant growth as was reported by Evseeva et al. (2018). These authors demonstrated that LPS from *A. brasilense* Sp245 specifically stimulated the regenerative capacity of wheat culture tissues and increased cell division in the wheat root meristem (Evseeva et al. 2011). In contrast, LPS from plant pathogens carry pathogen-/microbe-associated molecular patterns (PAMP/MAMP) that are recognized by the immune system and activate defense responses (Kutschera and Ranf 2019). Whether the same molecular mechanism is involved in sensing and transducing stimulatory effects by different LPS requires more investigation.

Based on studies of recognition of LPS by mammal cells, plants likely carry cellular receptors for LPS (Kagan 2017). However, whether plant cells can sense LPS from beneficial and pathogenic bacteria using the same receptor is unknown, as is whether plant cells have different receptors and different molecular components that respond to LPS from different bacteria. Information regarding the presence of receptors to LPS in plants is emerging, and will likely

Table 1 Effect of LPS and calcium on plant growth and POD activity

Treatment	Total length (cm)	Total fresh weight (mg)	POD activity (mMol/min/µg prot)
Control	19.15 ± 2.50b	134.40 ± 14.53ab	7.90 ± 0 .89bc
Sp245 LPS	21.40 ± 2.00a	148.70 ± 12.66a	8.62 ± 0.83ab
PAO1 LPS	18.78 ± 2.10bc	148.40 ± 21.39a	10.86 ± 0.25a
$LaCl_3$ (0.5 mM)	10.85 ± 2.30d	75.10 ± 15.30d	3.30 ± 0.47de
+ Sp245 LPS	9.50 ± 1.60de	53.70 ± 11.92d	2.36 ± 0.23e
+ PAO1 LPS	12.85 ± 2.00d	109.00 ± 18.70c	3.84 ± 0.94de
$CaCl_2$ (100 µM)	20.50 ± 2.40a	139.40 ± 10.10a	12.02 ± 0.94a
+ Sp245 LPS	13.30 ± 1.30d	105.00 ± 09.43c	5.78 ± 1.18 cd
+ PAO1 LPS	16.00 ± 1.90c	111.90 ± 24.62bc	7.73 ± 1.20bc

Data are mean ± of three independent experiments ($n = 10$). Letters indicate significant differences according to Duncan test ($p < 0.05$)

address this question. Although there are no reported receptor candidates that could sense LPS from PGPR, the lectin S-domain receptor kinase AtLORE was identified that could sense LPS from *Pseudomonas* spp. and *Xanthomonas* spp., a pathogenic bacteria. This putative receptor appears to specifically sense some LPS, but not LPS from *Escherichia coli*, *Salmonella enterica* or *Burkholderia* spp. and the receptor may even be specific to the Brassicaceae plant family (Ranf et al. 2015). Another interesting possibility by which plant cells distinguish different types of LPS is that plant receptors may perhaps recognize different structural components among different LPS. We have studies underway to address this question.

Author Contributions AAHE conducted the experiments. ECM contributed with technical assistance to experimental setup. AAHE and EGP discussed the results. EGP was the author of project planning and wrote the manuscript.

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Compliance with Ethical Standards

Conflict of interest On behalf of all authors, the corresponding authors state that there is no conflict of interest.

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